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Scaffold Materials Based on Fluorocarbon Composites Modified with RF Magnetron Sputtering

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1. Introduction

A bone is a remarkable organ, which plays the key role in performing of such critical functions in human physiology, as protection, movement and support of other organs, blood production, accumulation and homeostasis of minerals, regulation of blood pH, and location of many cells – progenitors (mesenchimal and hemopoetic). The importance of a bone becomes clear in the case of such diseases, as osteogenesis imperfecta, osteoarthritis, osteomyelitis and osteoporosis when a bone is not functioned by proper way. These diseases together with traumatic injuries, orthopaedic operations (total joint replacement, spine arthrodesis, implant fixation and other) and the first tumor resection lead to formation of bone defects. Clinical and economical aspects, accompanying treatment of bone defects are bemusing (Porter et al., 2009). For example, quantity of total joint arthroplasties (TJA) and revision operation only in USA increased from 700 000 in 1998 up to over 1.1 million in 2005 (American Academy of Orthopedic Surgeons, Web Site). According to estimations of specialists medical expenses connected with fractures, reimplantations, and replacement of hip and knee joints to 2003 exceeded \$ 20 billions and to 2015 will exceed \$ 74 billions (American Academy of Orthopedic Surgeons, Web Site; Kurtz et al., 2007). Traumatic bone fracture caused about 8.5 million doctor's appointments and led to about 1 million hospitalizing (American Academy of Orthopedic Surgeons, Web Site). Also, in 2005 there were performed more than 3000 pediatric hospitalizations connected with cancer with cost more than \$ 70 million (US Department of Health and Human Services, Web Site).

In the cases of non-union or defects of critic sizes it is necessary to use replacing materials for filling of bone defects. Actual gold standard of treatment of bone defects of critical sizes is transplantation of autogenous bone. At such treatment the host bone is removed from other part (usually from pelvis or ilium crest) and used to fill the defect. However, complication rates at transplantation of autogenous bone exceeds 30 % and can include morbidity of a donor site, pain, paresthesia, long hospitalization and rehabilitation, higher danger of deep infection, hematoma, inflammation and limited legal capacity (Silber et al., 2003). Other rational version for patients and surgeons is use of bone tissue of other people (usually cadavers) named allograft which can be obtained both from viable and sterilized nonviable sources. During last years many orthopaedic procedures connected with use of allografts have been performed in various countries. Success of autograft and allograft

procedures is described to physical and biological similarity of donor and host tissue. However, orthopaedic allografts bears danger for a donor to obtain infection (case rate is more than 3 %) (Mankin et al., 2005) and for a host – transferring of diseases and immune responses (Nishida & Shimamura, 2008). It is possible to consider as acceptable option for patients demanding restoration or replacement of bone use of xenografts or un-human tissues. Each success in xenotransplantation of various cells, tissues and organs, caused optimism and social acceptance of this growing technology (Lanza et al., 1991). However, results of clinical investigations, being performed during more than 20 years, led scientists to conclusion about unsuitability of xenografts for restoring surgeries owing to real and recognizable danger of transfer diseases or viruses, infections, toxicity, connected with sterilization, immunogeneity and final rejection by the host (Laurencin & El-Amin, 2008).

To solve these problems, it has been proposed so-called tissue engineering approach, which became usable alternative allowing stimulating regenerative ability of the organism – host (Kanczler & Oreffo, 2008). To realize this approach on practice many investigators are developing various complicated synthetic construction, named bone scaffolds which simulate complicated physical-chemical properties of bone. Use of such synthetic bone scaffolds is connected with such qualities as wide availability of materials for their manufacturing, elimination of the danger of transfer diseases, decrease of quantity of surgery procedures and reduction of infection danger or immunogenicity.

Basic conception, laying in the basis of tissue engineering is use of natural biologic response on tissue damage with high technological principles. Since role of cell signaling and functionality are displayed in tissue engineering with great clearness, specialists in the field of tissue engineering develop multifunctional bioactive scaffolds. Ideal synthetic scaffolds have to provide certain physical – chemical milieu in the time of biodegradation, while stimulating actively desirable physiological response and preventing undesirable one (Lee & Shin, 2007).

To fulfill these biomimetic requirements a synthetic bone scaffold must:

1. provide temporary mechanic support for affected area,
2. act as substrate for deposition of osteoids,
3. have architecture which allows flowing processes of vascularization and in-growth of bone,
4. promote migration of bone cells in a scaffold,
5. support and promote osteogenous differentiation in a non-bone synthetic scaffold (osteoiduction),
6. enforce cellular activity in direction of scaffold – tissue host integration (osteointegration),
7. degrade by controlled way to easy load transfer to developing bone,
8. generate non-toxic degradation products,
9. not provoke active confirmed inflammation response,
10. survive sterilization without loss of bioactivity,
11. deliver bioactive molecules or drugs by controlled way to accelerate healing and prevent pathology.

To satisfy above mentioned criteria such various strategies of tissue engineering, as cell transplantation, cell-less scaffolds, gene therapy, stem cell therapy and growth factor delivery were applicated (Ki et al., 2008; Leeuwenburgh et al., 2006; Kumar et al., 2003; Schneider & Decher, 2008; Kim & Mooney, 1998). On practice majority of bone tissue

engineering approaches uses combination of these strategies. However, following two main strategies of cell engineering are considered as the most perspective (Langer & Vacanti, 1993):

the first one - mesenchymal stem cells (MSC) are isolated (usually from a patient) before implantation, in following they are expanded *ex vivo*, seeded on a synthetic scaffold, where they obtained possibility to produce extracellular matrix (ECM) on the scaffold and finally, implanted in bone defects or cavities in patient tissues (see Fig. 1) (Mistry & Mikos, 2005); the second one - a cell-less scaffold is implanted immediately after damage / resection of bone.

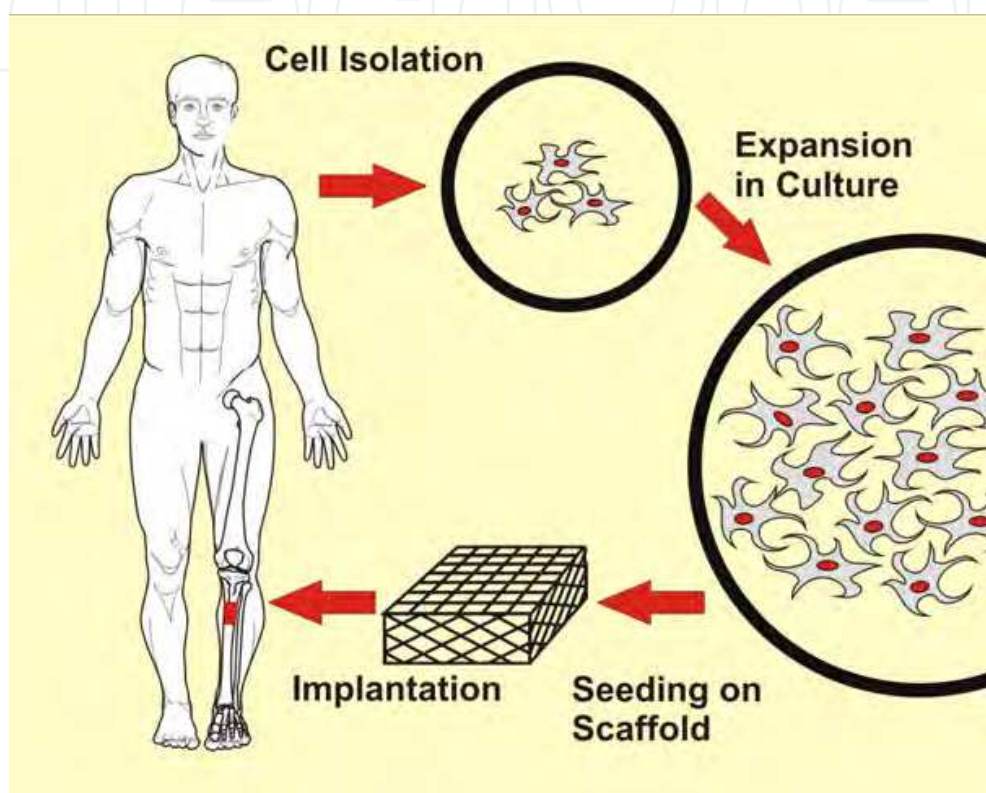


Fig. 1. Scheme of bone tissue engineering by means of scaffolds seeded with cells cultured before implantation *ex vivo*

MSC are pluripotent cells being able to differentiate in cells of various types. Under action of such chemical agents as dexametasone, ascorbic acid, and β -glycerol phosphate differentiation of MSC may be directed in side of bone forming cells or osteoblats, which than produce bone ECM within a scaffold *ex vivo*. It has been shown in many preclinical experiences that MSCs increase osteogenous ability and are integrated with native tissue more quickly than cell-less scaffolds (Service, 2000).

In spite of huge potential of this approach to bone cell engineering, it is necessary to overcome many barriers to transfer it in clinical practice. The first and most considerable one is defined by the fact that in number of investigation it has been shown that MSCs, which were cultured extensively *ex vivo*, being implanted *in vivo*, loss their phenotypical behavior (such as osteodifferentiation and bone forming ability) (Banfi et al., 2000). The second problem appearing at this approach is connected with comparable low concentration of MSCs in bone marrow and their characteristic low proliferative ability and consists in hardship of obtaining of sufficient cell density in a large scaffold (Bruder et al., 1997). In

addition to higher risk owing to necessity of the second operation surgeons are facing with necessity to state strict measures of sterilization for scaffolds seeded with cells cultivated *ex vivo* within period up to some weeks.

The second main approach of tissue engineering includes implantation of a cell-less scaffold immediately after trauma/resection of bone (see Fig. 2). Guiding principles of such approach are the same of the first one. However, to provide rapid healing, designing of a scaffold which is built in the native bone tissue and is capable to promote migration of local MSCs in scaffold, to support and promote osteodifferentiation (osteinduction), while providing formation of biodegradable matrix, which increase production of ECM by MSC and eventually integrates with native tissue and fills cavities or defects (osteointegration) is more critic (Nair & Laurencin, 2006). Obvious values of this approach are that cell-less scaffolds can be sterilized more easy, have large shelf time and lowest potential of infection or immunogenicity.

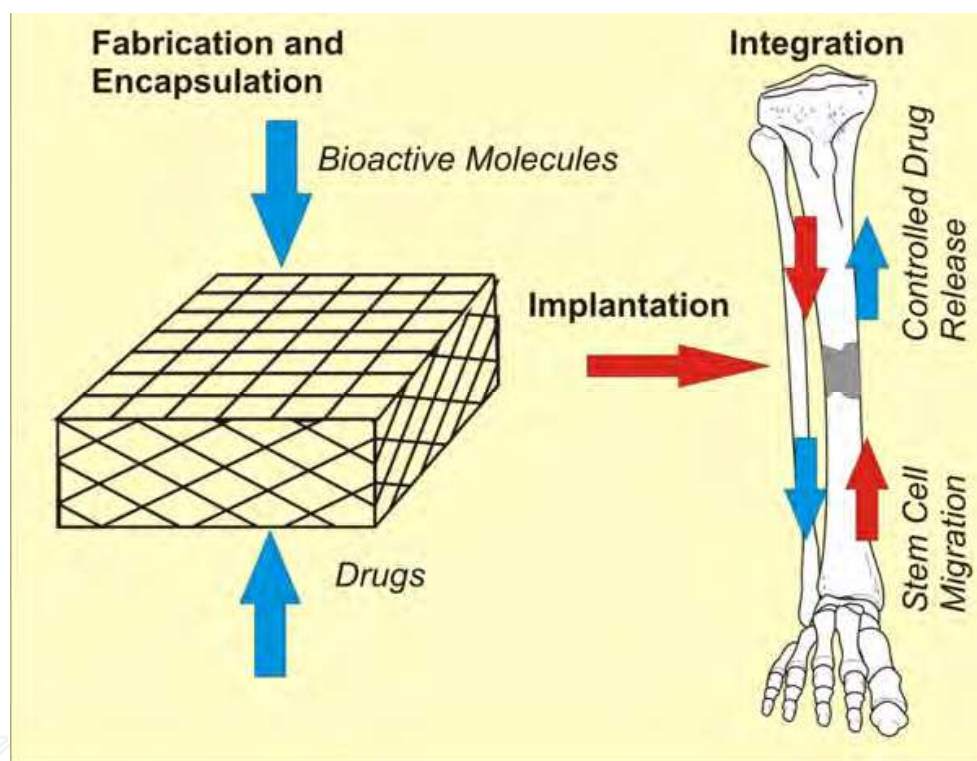


Fig. 2. Schematic description of the second strategy of bone tissue engineering in which biological molecules and pharmaceutical agents are encapsulated in cell-less scaffold to release after implantation

The further development of these strategies is designing of porous scaffolds for bone tissue engineering taking into account not only biomechanics and achievements in the field of material sciences, but and understanding of processes running both in damaged bone on the each stage of its regeneration and on tissue – implant interface. Such constructions must have porous structure, allowing inserting in them pharmacological preparations and cellular materials which will be released from construction while stimulating and controlling osteogenesis processes therefore they can be named biochips. Preliminary consideration of concepts for use of biochips for tissue engineering it is possible to find in papers (Santini et al., 1999, 2000).

2. Methods of scaffold manufacturing

By their essence a biochip is a porous scaffold loaded with bioactive agents (such as various growth factors, DNA, bone morphogenetic proteins etc.) with programmable time and space patterns (principles) of releasing of these components allowing stimulating purposefully osteogenesis process in the implantation place. Taking into account this fact, a biochip performs, in certain degree, function of a drug delivery system with scaffold as basis of his construction.

2.1 The role of physical impetus in synthetic bone scaffolds

Hierarchic geometrical structure of bone, shown in Figure 3 are critical not only for macroscopic mechanical properties, but and for survival of bone cells and functionality in micro- and nanoscale. Owing to directs deposition and bounding of extracellular matrix (ECM) proteins and cell cytoskeleton through cell receptors cells sense and respond to matrix physical properties converting mechanical impetuses in intracellular chemical signals which initiate such activity as gene expression, protein production, and general phenotypical behavior (Vallet-Regí et al., 2006).

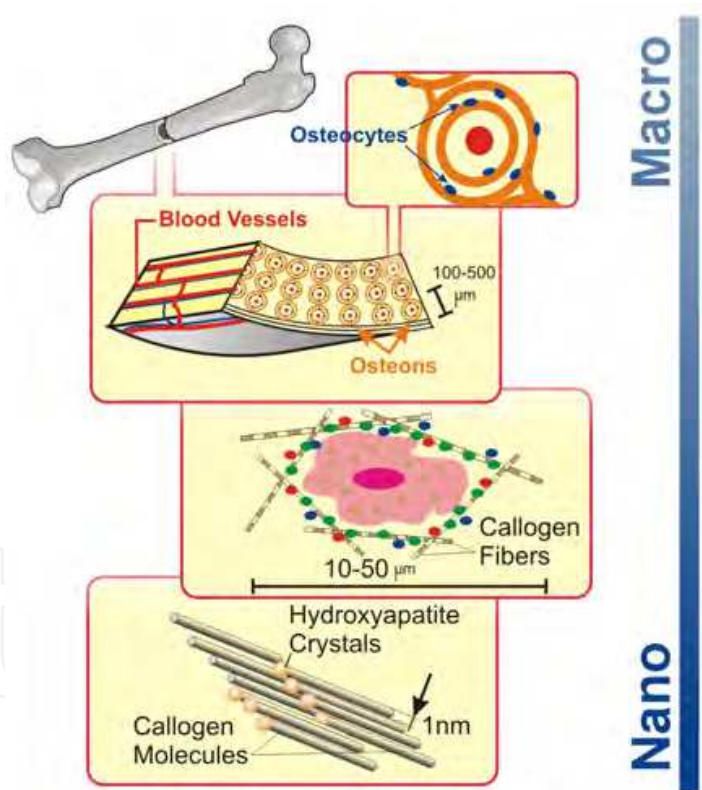


Fig. 3. Scheme of sophisticated hierarchical structure of bone

Therefore the main aim of bone scaffold designing is simulation of unique bone micro- and nanoscale characteristics. It is shown in literature that bone cells are influenced considerably by topography *in vitro* (Desai, 2000). Such, Vagaska found that osteoblasts grown on the microrough surfaces have been stimulated to differentiation as it is shown by expression of their genes and higher mineralization level in comparison with cells growing on smooth surfaces (Vagaska et al., 2010).

Microscale bone peculiarities provide channel for vascularization, nutrient delivery, and cell migration. Some investigators supposed that to assure migration of bone cells and nutrients in a scaffold it is necessary to have pores with sizes closed to cell sizes (Mo et al., 2004). High porous microscale scaffolds also allow more high levels of nutrient diffusion, vascularization, and improved spatial organization for cell growth and ECM production (Woodard et al., 2007). However, some ambiguousness remains in relation to optimal porosity and pore sizes for 3D bone scaffold. Review of literature sources shows that pore sizes in range of (10 – 400) μm can provide sufficient intake of nutrients and osteoblast cells while keeping structural integrity (Walsh et al., 2003). The wide variety of methods of manufacturing of biochip matrix frameworks was investigated to recreate microscale porosity and special organization of native bone. Some examples of good developed methods include: microfabrication, photolithography, calcium phosphate sintering, rapid prototyping, mold extrusion, salt leaching, phase separation, fiber bounding, membrane and polymer delamination. Surprising result is that the most successful methods of fabrication include emulsion and sintering of calcium phosphate materials. Synthetic bone scaffolds with porosity up to 70 % have been prepared using these methods. These scaffolds have demonstrated excellent bioactivity *in vitro* and in-growth of bone *in vivo* (Christenson et al., 2007).

However, microscale porosity plays a key role in scaffold osteoconductivity, nanoscale architectonics of material acts basic physical influence on osteoinductivity and osteointegration of a scaffold. Up to now majority of methods of manufacturing of synthetic bone scaffolds were limited macro- and microscale and were impossible to recreate sophisticated bone nanoarchitectonics. However, in the last decade some successes have been achieved in nanofabrication and, consequently, in control of cell behavior by means of nanomanipulation. Bone cell in native tissue interact with nanoscale proteins and minerals. It is stated authentically, that all living systems are controlled by molecular interactions in nanometer scale. Unique properties of all molecular constructive blocks of life, such as proteins, lipids, carbohydrates and nucleic acid, are controlled by their nanoscale sizes and patterns. Consequently, bone cells are predisposed to adhere, grow, proliferate, differentiate and to product ECM on the basis of nanoscale interactions (Horbett, 1994.). In addition to increase cellular activity nanoscale materials can have regulable surface area and surface energy which, as it was shown, influence on adhesive protein adsorption. The wide range of methods of scaffolds nanofabrication has been developed likewise to microscale fabrication. Examples of successful methods of nanofabrication of 2D and 3D objects include electrospinning (Ki et al., 2008) electrostatic spraying deposition (Leeuwenburgh et al., 2006), RF plasma spraying /deposition (Kumar et al., 2003), molecular self assembling (Schneider & Decher, 2008).

2.2 Methods of 3D- porous scaffold fabrication

Porous ceramic degradable scaffolds are used mainly for correction of bone tissue defects, both gained, as result of trauma of operation, and inherent. Taking into account low mechanical strength of porous ceramics matrices, they are used in areas not bearing load, for example, in maxillofacial surgery. The basis for creation of porous ceramic degradable matrix is hydroxyapatite (HA), obtained both from clear reagents, and by means of hydrothermal treatment of aragonite.

2.2.1 Coral scaffolds

Aragonite is a skeleton of sea reef-forming corals *Porites*, from which porous scaffolds are manufactured by means of hydrothermal treatment in presence of ammonium phosphate at temperature about 275 °C and pressure of 1200 psi by reaction (Kim & Mooney, 1998):



The carried out investigations revealed two coral kinds having pore size suitable for transformation in hydroxyapatite and following use as matrices for tissue engineering (Ki et al., 2008). It has been found that porosity characteristics of sea invertebrates depend on type and kind. Within kinds and between members of these kinds mean pore diameters are quite uniform with low variation range by a structure.

Before exchange reaction organic component of these corals are removed by sodium hypochlorite. Extreme chemical and thermal conditions of exchange reaction destroy any remnant organic material. Structure of obtained hydroxyapatite is almost accurate replica of porous skeleton of sea animals including their interconnecting porosity with pore diameter 150 – 500 µm resembling the system of haversian channels of compact human bone, that promotes growth and differentiation of cells leading to rapid ingrowth of bone tissue at implantation (Jones & Hench, 2003). Ingrowth of connective and bone tissue in such matrix is accompanied with moderate inflammation and giant-cells reactions (Massry & Holds, 1995), minimal capsule formation about matrix (Dutton, 1991) and osteogenesis in coral depth (Holmes, 1979). The mechanism of osteoinducing action of HA is not revealed decisively.

Ingrowth of bone tissue also depends on pore size and porosity which determine total contact area of porous matrix surface with surrounding tissues. This index determines not only velocity of penetration of biologic liquids and biodegradation of material, but is important element determining ability of bone cells to seed an implant. So, work surface area of dense and porous HA was from 0.01 to 1.0 m²/g, accordingly (Tofe et al., 1993).

In macroporous material with pore diameter of 20-200 µm natural liquid convection appears owing to capillary processes. It promotes transportation of substances, biodegradation or dissolving of material (Walsh et al., 2006). So, at using of corals with through porosity, process of cell migration and osteoclast resorption of material lasts about 6 - 8 weeks. If pores are not interconnective, closed cavity appears from which exit of substances is hampered. In these conditions process of biodegradation can delay up to 18 months (Holmes et al., 1984; Hanusiak, PhD Thesis).

To simulate osteon – free stroma of cortical bone one uses the coral *Porties* skeleton. Solid frameworks and porous network are continuous and interconnective domens. Mean size of solid components of implant framework is 75 µm, mean pore diameter achieves 230 µm and their interconnections 190 µm, volume part of cavities is 65 % (White & Shors, 1986).

To simulate osteon – free stroma of cancellous bone one uses the coral *Goniopora* skeleton. Mean size of solid components of implant framework is 130 µm, mean pore diameter achieves 600 µm and volume part of cavities is 63 % (Malluche et al., 1982).

Due to high biocompatibility of a coral, a material obtained from coral skeletons has been permitted for clinical use in the USA only in four years after first implantation performed by A. C. Perry in 1985. In period of six years coral spheres have been implanted in 25,000 patients. Wide distribution of the materials is promoted by industrial production of coral apatite matrices by the firm «Interpore Int, California» (USA) under trade names Interpore®

200, ProOsteon®200, ProOsteon® 500, and «BIO-EYE» by the «Integrated orbital implants Inc.» (USA).

2.2.2 Scaffolds from cattle bones

Materials obtained from cattle bones are used for fabrication of matrices, too. They are unsintered (BioOss®, Oxbone®, Lubbock®, Laddec®) or sintered at temperature about 1000 °C (BonAP, Endobon®, Osteograf®). To remove proteins unsintered materials are treated usually in organic solutions or CO₂ supercritical liquids. Remained bone material is mainly carbonated hydroxyapatite (CHA) with little additions of magnesium, sodium and other trace elements. Its chemical composition and crystallinity, naturally, resembles one of a bone with large specific surface area and macroporosity (Thaller, 1993). This material has osteoinductive properties and at implantation in bone defects bone formation takes place on its surface. Antigenic or immunogenic inflammation responses are not observed while using of deproteinized cattle bone, however, velocity of its resorption is issue of disputes. In some investigations it has been observed no signs of resorption (Valentini et al., 1998) or only slight resorption (Piatelli et al., 1999). In other animal experiments vats resorption took place (Merkx et al., 1999). To burn organic components and to remain sintered body cancellous cattle bone is heated to temperature about 1000 °C. In dependence on conditions of sintering, temperature and time, porosity (micro/ macroporosity) of initial bone has been kept essentially, but phase composition is changed sharply, when carbonate apatite (CHA) is decomposed at high temperatures on HA and β-TCP (Le Geros et al., 1991). These materials differ by composition (HA/ β-TCP ratio) in dependence on stoichiometry of initial cattle bone.

However unique chemical composition and spatial pore structure of above mentioned matrices is combined with such their physical chemical properties as high density and fragility which create many inconveniences at work with them, since hinder hand treatment to model a scaffold. To work with them it is necessary additional equipment of operation room with cutting or milling diamond instruments. Impossibility of suture fixation of tissue to such scaffolds, their coarse rough surface injuring surrounding tissues (Goldberg et al., 1992; Kim et al., 1994), force in certain cases to wrap them with donor or synthetic tissue.

Therefore scaffolds from synthetic calcium phosphate are implemented actively as alternative to natural expensive material (for example, for orbital implants trade marks «FCI ophthalmics» and «LIFECORE»). Implant FCI (French firm Issy-Les-Moulineaux) of the third (last) generation has the same chemical composition and mechanical strength as natural coral, sufficient porosity and suitable for hand treatment.

2.2.3 Scaffolds from synthetic calcium phosphates

Synthetic calcium phosphates are divided on hydroxyapatite (HA), Ca₁₀(PO₄)₆(OH)₂ (Cerapatite®, Synatite®); tricalcium phosphate (β-TCP), Ca₃(PO₄)₂ (Biosorb®, Calciresorb®, Chronos®), bi-phase calcium phosphate (BCP) for admixtures HA and β-TCP (Biosel®, Ceraform®, Eurocer®, MBCP®, Hatric®, Tribone 80®, Triosite®, TricOs®); and unsintered or calcium deficit apatite.

Calcium phosphates have various solubility velocities *in vitro* or velocity of solution in acid buffers that can reflect comparative dissolution or degradation *in vivo* (Le Geros et al., 1995). Comparative solution degree is changed in following order: α-TCP >> β-TCP >> HA.

Calcium phosphate (CaP) biomaterials are available in various physical forms (particles or blocks, dense or porous). One of the main characteristics is their porosity. Ideal pore size for bioceramics resembles one of cancellous bone. Before sintering at high temperatures macroporosity (pore size $> 50 \mu\text{m}$) intentionally introduced by means of additions of volatile substances or porogens (naphthalenes, sugars, hydrogen peroxide, polymer pellets, fibers etc.) before sintering at high temperatures (Hubbard, 1974). Macroporosity forms when volatile substances are released. Microporosity is result of sintering process, where temperature and time are critical parameters. It was displayed that microporosity allows circulation of body liquids, while microporosity provides matrix for colonization of bone cells (Daculsi et al., 1990). It was reported, that mean pore size $565 \mu\text{m}$ is ideal macropore size for in-growth of bone in comparison with one of smaller size ($300 \mu\text{m}$) (Gauthier et al., 1999). The main difference between commercially available BCP is microporosity which depends on sintering process. For osteogenic or osteoconductive properties low temperature sintering processes have to conserve or to increase microporosity. Only bi-phase calcium phosphate prepared at low temperature (less than 1100°C) are both micro- and macroporous.

In spite of that synthetic calcium phosphates are cheaper than their natural analogues, they have essential shortcoming – fragility. Therefore in last decade porous polymers which are elastic, comparatively easy treated and modeled, fixed with suture material became to use as rival material for scaffolds.

2.2.4 3D porous polymer scaffolds

Various methods are used for manufacturing of 3D porous scaffolds. Usual methods include fibrous felt, fibrous bonding, mold casting, solution casting / particle leaching, gas foam forming/ particle leaching, phase separation and high pressure treatment. Examples of porous scaffolds are given in Figure 4.

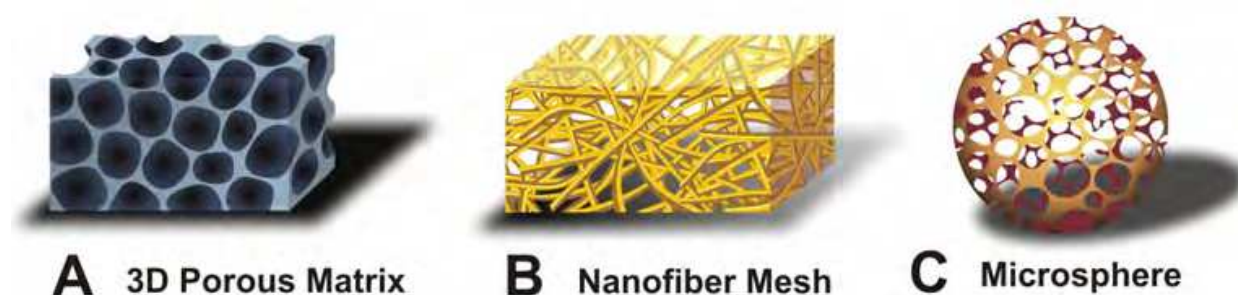


Fig. 4. Various forms of polymer scaffolds for tissue engineering: A – typical 3D – matrix in solid foam form, B – nanofiber matrix, C – porous microspheres

Fibrous bonding

3D porous scaffolds can be fabricated bounding polymer fibers in crossing point using a secondary polymer. For example, fibers of polyglycolic acid (PGA) can be bonded by casting of poly (L-lactic acid) (PLLA), cooling and following removing of PLLA (Whang et al., 1995). However this method faces with difficulties connected with control porosity and solvent selection.

Freeze-drying of emulsion

Freeze-drying of emulsion solution consisted from dispersed water phase and continuous organic phase containing biodegradable polymer can lead to formation of porous scaffolds with various size and interconnectivity of pores. While using this method, Mikos A.G. prepared scaffolds on the basis of poly (lactic-co-glycolic) acid (PLGA) with porosity up to 95 % and pore size up to 200 μm (Mikos et al., 1993).

Solution casting/particle leaching

Solution casting/particle leaching is, probably, the handiest method for preparation of porous scaffolds. It includes casting of mixed solution polymer – salt - organic solvent with following evaporation of solvent and dissolution of salt particles in water solution. However, this method has restrictions, since allows fabricating only thin membranes with dense surface layer as well as may contain remnant particles of the salt used in the time of process. Applied efforts allowed obtaining thin scaffolds with open cellular morphology and porosity higher 93%. To prepare thick 3D scaffolds, PLLA or PLGA porous membranes have been collected in multiple-layer structures of various anatomical forms (Mooney et al., 1996; Harris et al., 1998).

High pressure treatment

High pressure treatment also known as technology of supercritical liquid is performed by means of action on dry polymer with such high pressure gas as carbon dioxide which forms mono-phase solution polymer/ gas. Then pressure is discharge to form thermodynamic instability of dissolved CO_2 that leads to nucleation and growth of gas vesicles, created pores within polymer matrix. Yoon J.J et al. used this method to create high-porous PLGA sponges (Yoon & Park, 2001). Solid PLGA disks, prepared by compressing casting or solution casting, were saturated with CO_2 under high pressure, then pressure was discharged and formed macroporous structure. The main quality of this method is that it eliminates using of organic solvents. Since this method does not include heating process, it is useful for incorporation of thermosensitive bioactive agents. Porous structure is quite uniform since CO_2 – gas uniformly dissolved within polymer as well as acts as softener which induces denser packing of polymer chains that increases mechanical strength. However, this method leads to insufficient interconnectivity of pores within scaffold and in many cases to non-porous surface. To eliminate this shortcoming Nam Y.S. et al. modified this method combining it with method of particle leaching (Nam et al., 2000). Mixture PLGA/NaCl has been casted under pressure in solid disks which were exposed to action of CO_2 gas and submerged in water for leaching of a salt. Described process led to formation of high interconnected mesh without signs of non-porous surface.

Gas foam formation/particle leaching

The method of «gas foam formation» had been developed by Park group, using effervescent salt as gas foaming agent. Double PLA–gel solvent blend containing particles of dispersed salt of bicarbonate ammonium was poured into a mold and then was submerged in hot water. Releasing of gaseous hydrides of nitrogen and carbon dioxide from solidifying polymeric matrix led to the formation of high interconnected pores. Formed scaffolds had open macroporous cellular structure with uniform distribution of pores which size ranged from 100 to 200 μm and without signs of surface layer. Method of gas foam formation / salt releasing had been improved further for preparation of PLGA scaffolds with addition of

citric acid in water solution (Kim et al., 2006). In this case the amorphous PLGA dissolved in chloroform was precipitated in ethanol to obtain gel suspension. Particles of ammonium bicarbonate mixed with this gel paste were poured into a mold and semi solidified at room temperature. After that they were submerged in an aqueous solution of citric acid. This method allows obtaining macroporous PLGA scaffolds with porosity more than 90 % and pore size about 200 μm . Porosity and mechanical strength can be controlled by regulating degree of gas forming reactions basic – acid between two salts. These scaffolds were commercialized in Republic Korea under trade mark Innopol-D™ for plastic surgery application. The same principle of gas foam formation allows manufacturing injector PLGA scaffolds – microforms, while using method of evaporation of solvent from double emulsion (Nam & Park, 1999). High opened porous microsphere with size of (200 – 300) μm were prepared by insertion of ammonium bicarbonate in drops of inner water phase, which formed actively gas vesicles in process of removing of solvent. Surface pore size achieved 30 μm , being sufficient for infiltration and seeding that was displayed by culturing of fibroblasts.

Thermally induced phase separation

Method of phase separation is based on thermodynamical separation of uniform polymer – solvent solution on the phase reached with polymer, and the phase depleted with polymer. Usually separation are performed either by action on the solution of other immiscible solvent or cooling of solvent to the point under two node solubility curve (Nam & Park, 1999). In particularly, thermally induced phase separation (TIPS) uses thermal energy as latent solvent to induce phase separation. Polymer solution is quenched under freezing point and than freeze drying forming porous structure, which parameters can be fine controlled, while regulating various thermodynamical and kinetic parameters. The most early scaffold for tissue engineering prepared by TIPS method had microporous structure (1 – 10) μm without interconnectivity and open cellularity. Park used TIPS method to obtain scaffolds with macroporous structure and open cellular morphology. To increase size of phase separated drops one used roughness increase process that increased pore size up to 100 μm . Obtained scaffolds had pores with uniform distribution and porosity more than 90 %, too. They also displayed that addition of surfactant (Pluronic F127) increased scaffold pore morphology.

Electrospinning

Owing to its simplicity and efficiency electrospinning is the most wide used method for manufacturing of nanofibrous unwoven matrices which is considered the most prospective strategy to manufacture nanofibrous scaffolds simulating bone. Popularity of electrospinning is stipulated, to all appearance, by simplicity of the experimental plant, ability to include bioactive molecules and plasticity which it provided. Investigation both *in vitro* and *in vivo* has demonstrated that cell osteopredecessors differentiate, proliferate and adhere to synthetic nanofibrous matrices. In electrospinnig process (see Fig. 5) polymer melting or solution in organic solvent are spread out nozzle under action of gravity force and/or mechanical pressure combined with strong electric field created by high voltage (10 – 20) kV. When force acting on electric load of the material exceeds surface tension of polymer solution drop, polymer jet arises from which solid nanofibers are formed at following evaporation of solvent (Li et al., 2002). Electrospinning allows obtaining nanofibers of such materials as biodegradable polymers, for example: PLGA and

polycaprolacton (PCL), materials soluble in water, for example poly (ethylenoxide) (PEO), polyvinyl alcohol (PVA) and such natural polymers as collagen, silk protein and other peptide.

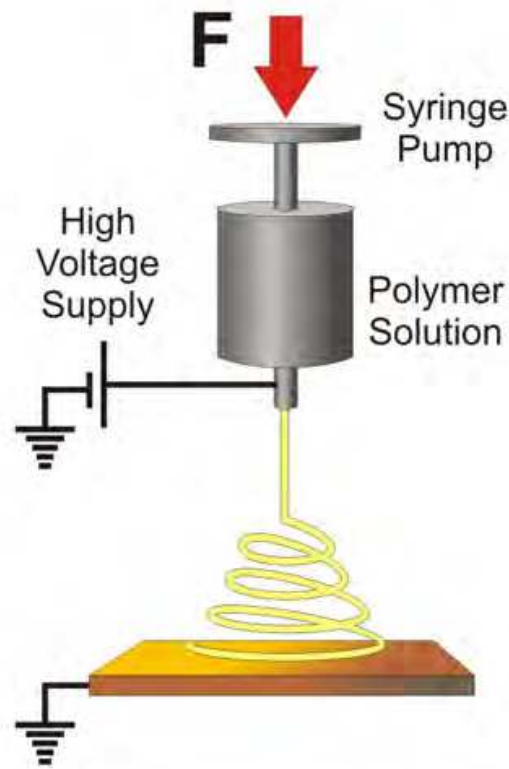


Fig. 5. Typical electrospinning plant consisting of high voltage supply, syringe and syringe pump

Usually electrospinning is used for creation of 2D mesh structure with nanosized pores, which are insufficient for seeding and infiltration of pores. Therefore it is necessary to form macroporous and nanofibrous 3D hybrid scaffolds with required volume and form for application as implantable scaffold for regeneration of tissues.

2.3 Methods of polymer scaffold modification

Although porous scaffolds with good interconnected pores are sufficiently suitable to allow infiltration and in-growth of cells such their surface characteristics as hydrophilicity /hydrophobicity resulting from chemical composition can not be satisfied to induce selective adhesion, migration and proliferation of cell and, as consequence, osteogenesis. In majority of cases specific cellular interactions are required to form required tissue. In general, adsorption behavior of proteins with accompanying determining cellular interactions is determined by implanted biomaterials surface properties. To achieve optimal osteogenesis number of investigators made attempts to simulate natural extracellular matrix by immobilization of naturally obtained biomolecules on polymer scaffold surface. Scaffolds with engineering surface were able to amplify adhesion and growth of cells or prolonged release of growth factors (Wolke et al., 1992), thereby assuring chance to facilitate process of tissue regeneration. Scaffold surface can be functionalized both by physical adsorption and by chemical modification.

Important stage of creation of matrix is modification of its surface with aim to create hydrophilic coatings. As experience shows, in the case of using of matrices for restoration of bone tissue defects the best way to provide it is coating the porous matrices surfaces with calcium phosphates, for example, hydroxyapatite. This coating provides accelerated integration of implants with surrounding bone tissue. Some methods are developed for realization of this process, which are used with large or small success. Possible methods of modification of polymeric scaffolds are given below.

2.3.1 Biomimetic deposition

Biomimetic deposition is process of crystallization, i.e. falling of crystals on matrix surface from oversaturated solutions containing calcium phosphates. Principally, biomimetic coatings are formed from solutions simulated body liquids. Advantage of this method are possibility of applying of high crystalline coatings, absence of complicated technological process, simplicity of technological tools, possibility to apply coatings on complicated geometrical forms at room temperature. It makes the method of biomimetic deposition suitable to apply CaP coatings on scaffolds. As shortcomings of the method it is possible to name low adhesion of CaP coatings, especially to inert polymer substrates, large time (about 8 – 10 days) necessary to apply coatings with thickness about some micrometers (Habibovic et al., 2002).

2.3.2 Plasma spraying

Plasma spraying is the method which is used the most often for application of CaP coatings on orthopaedic implants. The method is based on feeding of CaP particles in gas – carrier through plasma of electric or gas -fired arc. Under action of high temperature gas is ionized and becomes plasma, which is accelerated up to high velocities. CaP particles, transferred by gas – carrier, are heated, melted, than deposited on a substrate. However the method proved its usefulness, its shortcoming is low cohesion of coating, especially at applying of calcium phosphate layers of large thickness. In addition at applying of CaP by method of plasma spraying the substrate temperature increases considerably that makes this method practically unsuitable for applying of CaP ceramic coating on polymer materials, on consequence of their melting and thermal destruction (Chen et al., 1994).

2.3.3 The method of pulsed laser deposition

The method is based on process of rapid melting and evaporation of target material in vacuum under action of high energy laser radiation with following transition of evaporated material from target and its deposition on a substrate. The method of pulsed laser deposition is technologically flexible method since energy source – a laser – is located out the vacuum chamber and can be changed on any other source optimally suitable to evaporate a target of one or another chemical composition.

The method of pulsed laser deposition allows to evaporate practically each material, sequential evaporation of various targets with different chemical composition allows to deposit both mono-phase and multilayer films of various materials. Other advantage of pulsed laser deposition is keeping in coating of stoichiometric composition of target to be evaporated. Controlling laser generation mode, it is possible to regulate very accurately thickness of the deposited film (Fernández-Parada et al., 1998). As shortcomings of the method it is possible to name impossibility to obtain coatings of uniform thickness on spatial porous structures presented by scaffolds for tissue regeneration and low velocity of coating growth.

2.3.4 Pulse ion deposition method (deposition from ablation plasma formed by powerful ion beam)

The method of power ion beams (PIB) is based on process of rapid melting and evaporation of target material at its irradiation by power ion beams with energy of some hundred keV and following deposition of coating from formed ablation plasma.

The PIB method is characterized by high values of material coefficient of energy adsorption (for PIB coefficient of energy adsorption is about 1, for laser radiation ~ 0.1). Power efficiency of ion pulsed accelerators is (20 – 40) %, that exceeds considerably power efficiency of laser plants which does not achieve 3 %. The method allows obtaining nanosized multilayer coating on various materials: metal, alloys and ceramics. Coating, obtained by PIB method are amorphous, characterized low inner stresses and good adhesion to a substrate being (10 – 60) MPa (Saltymakov et al., 2010; Struts et al., 2011).

However it is necessary to note that the method requires certain improvement and at the present this circumstance hinders wide use for formation of coatings on polymer scaffold.

2.3.5 The method of explosive evaporation

We proposed to use the method of explosive evaporation for applying of coating on metallic and ceramic implants. This method is based on phenomenon of instant evaporation of powdery material at its hit on the high temperature evaporator. Appearance of vacuum work chamber in process of coating applying is given in Figure 6. Structurally devices for explosive evaporation are manufactured by such way, that material particles would fed with velocity equal to velocity of its evaporation. In this case at steady conditions there are all components of material of complex composition will be in vaporous form in the same ratio as in initial material and the film of specified composition will be obtained on matrix surface. Advantage of the method is high velocity of calcium phosphate coating applying being about 5 $\mu\text{m/h}$ (Tverdokhlebov et al., 2010).

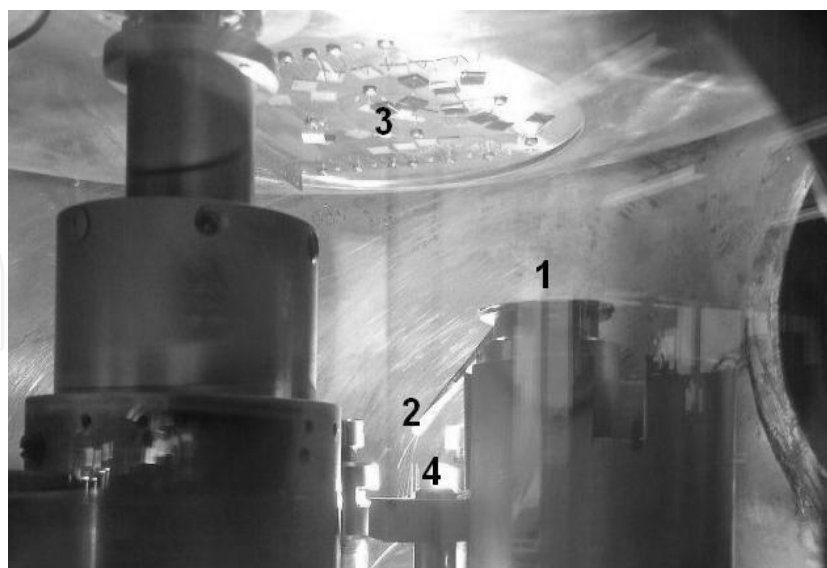


Fig. 6. Appearance of vacuum chamber of explosive evaporation plant: 1 – vibrating bin, 2 – device for feeding of materials, 3 – sample, 4 – evaporator

To estimate ability of application of the explosive evaporation method for applying of calcium phosphate coatings on polymer materials is necessary additional investigations.

2.3.6 The method of radio-frequency magnetron sputtering

Radio-frequency magnetron sputtering (RFMS) is used widely in microelectronics to apply films of complex chemical composition without change of their stoichiometry. The method is based on sputtering of material in vacuum owing to bombardment of target surface with work gas (mainly argon) ions, forming in plasma of anomalous glow discharge at applying of magnetic field.

Principal electric circuit of RF magnetron sputtering system is given in Figure 7. For applying of calcium phosphate coating one uses RFMS plants with work frequency, as rule, 13.56 MHz, allowing obtain high homogeneity of plasma and, as result, to achieve even applying of coating with growth rate of dielectric fields (0.2 – 0.8) $\mu\text{m}/\text{h}$.

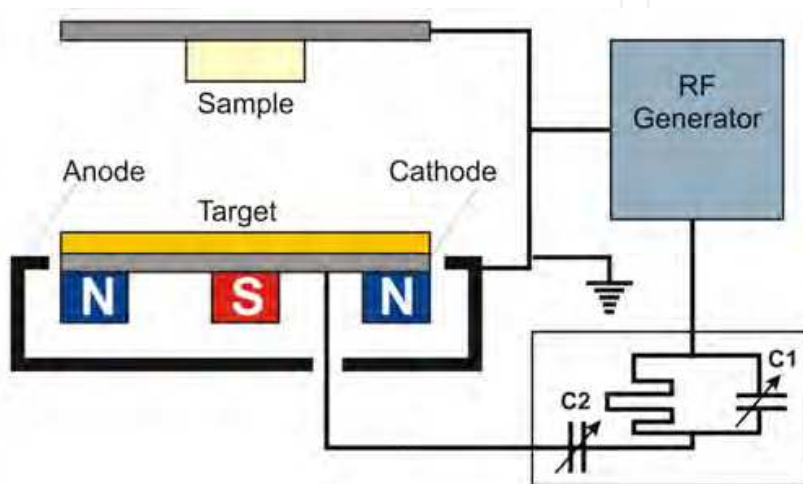


Fig. 7. Principle electric circuit of RFMS

RFMS technology allows to form elastic CaP coating with regulated chemical composition with thickness about 1–2 μm , low porosity, high adhesion to matrix material. Magnetron coating applied on polymer materials are able to withstand essential mechanical deformation without destruction. Microscopic investigation show that coating obtained by the RFMS method are solid, able to repeat initial matrix surface morphology, have no own macro- and microporous structure that is explained by mechanism of their atomic growth (Pichugin et al., 2008; Aronov et al., 2008). At the present the method of RF magnetron sputtering of calcium phosphate on polymer materials is developed by us in the maximal degree therefore it was used in this work for modification of scaffold surface.

3. Fluorocarbon composites modified with rf magnetron sputtering

As it has been shown above up to now treatment of diseases and injuries of loco-motor apparatus is the complicated clinical problem. Traditional methods and ways based on the use of inner and external osteosynthesis with application of fixators achieved limits of their biomechanical abilities.

For effective treatment of loco-motor apparatus injuries it is necessary to act actively on bone tissue and to control processes of its regeneration and mineralization. With this aim some scientific collectives proposed concept, based on active influence on processes of reparative osteogenesis by means of application of osteoplastic materials (in the first time – calcium phosphates, for example, hydroxyapatite (HA) and tricalcium phosphate (TCP))

and various growth factors (bone morphogenetic protein, fibroblast growth factor, transforming factor, platelet factor, insulin-like factors and others) which jointly promote processes of remodeling and regeneration of bone tissue (Karlova & Shachov, 2001; Barinov & Komlev, 2005).

It is necessary to note that variety of clinical problems to be solved with help of scaffolds stipulates various action on a scaffold and biomechanical characteristics of adjoining bone tissue (density, elasticity modulus and rigidity) and determines various requirements put to physical – mechanical characteristics of coating (thickness, porosity, crystalline structure, chemical composition, solubility etc.). By these causes for successful restoration of locomotor apparatus functions in various clinical cases traumatologists must have in their arsenal wide line of scaffolds with various surface characteristics which are suitable by optimal way for treatment of concrete patient.

However it is necessary to understand clearly that at the present it is impossible to manufacture, while using any one technology, a material which would totally satisfy the various and contradictory requirements put forward to scaffolds. It is evidently that to manufacture really efficient devices it is necessary to integrate various technologies of manufacturing of scaffolds with technologies of their surface modification. The paper is devoted to technology of hybrid scaffolds fabrication developed by our scientific collective in the Tomsk Polytechnic University (TPU).

3.1 Materials and methods of investigation

As binding material for manufacturing of composite scaffolds we selected copolymer tetrafluoropolyethylene with vinylidenefluoride (TFE/VDF). Our selection was stipulated by its high chemical resistance, good physical-mechanical characteristics, and temperature stability (Kataeva et al., 1975), and biological inertness (Grafskaya, 1967). Such polymers can be processed from solution that simplifies apparatus realization of framework manufacturing process.

As biologically active filler which assures realization of processes of osteoinduction and osteoconduction, we selected hydroxyapatite obtained by means of high temperature processing of biological raw materials. Selection of hydroxyapatite as biological active filler is not occasional but is stipulated by its high ability to bind various molecules including proteins, enzymes, antibody fragments, nucleic acids and others.

It is stated that level of protein bounding depends on phase composition of hydroxyapatite. So, high crystalline hydroxyapatite (crystallinity more than 90%) adsorbs about 90 ng/ml of protein and low crystalline (up to 60%) - 60 ng/ml. It is stated also that high crystalline CaP has more high ability to support growth of osteoblast cells in comparison with more amorphous form as well as control plastic surface in the *in vitro* system (Melican et al., 1998). Consequently, osteoinductive properties of hydroxyapatite in a large extent depend on its crystallinity, therefore task to obtain hydroxyapatite with set phase composition is urgent.

One of the ways to control crystallinity of calcium phosphates and, particularly, hydroxyapatite is method of high temperature burning of biologic raw materials, for example, cattle bones. Hydroxyapatite, obtained in process of such high temperature treatment after multiple washings is non toxic, apirogenic and sterile that makes possible to use it as filler to manufacture composite scaffolds. Other considerable advantage of the high temperature treatment method is keeping of microelement composition of processed biological raw materials.

The main task of this stage of investigation was study of action of temperature of biological raw material treatment on phase composition and parameters of crystalline lattice of hydroxyapatite by method of X-ray phase analysis. Investigations have been carried out with a Shimadzu XRD 6000 diffractometer using $\text{CuK}\alpha$ radiation. Analysis of phase composition, sizes of coherent scattering areas, inner elastic stresses has been performed while using PCPDFWIN data bases, as well as program of total profile analysis POWDER CELL 2.4.

Process of manufacturing of hybrid scaffold conditionally can be divided on 2 stages: manufacturing of frameworks and modification of its surface. Framework material was prepared on the first stage as solution of TFE/VDF copolymer in acetone at continuous stirring and constant temperature about 70 °C. Biologically active filler presenting HA dispersion was prepared by means of agitation of HA powder in ethyl acetate in a ball mill at 30 °C during 2 hours. After obtaining of TFE/BDF copolymer solution HA dispersion was added to it at continuous stirring up to achievement of necessary viscosity. Framework material was formed by the method of thermally induced phase separation, while using thermal energy as latent solvent to form porous structure of scaffold. After forming scaffolds specimens were placed in the cabinet dryer with temperature of 130 °C for 12 hours to remove solvent remnants. Selected modes allows to obtain homogenous and flexible (elastic) frameworks TFE/VDF-HA from components prepared with mass ratio of TFE/VDF polymer to HA filler being equal 30:70.

Surface morphology of TFE/VDF-HA frameworks are studied by means of optical microscopy with the «Motic» microscope with following computer processing of data with help of the Motic Image Life plus program package, which allows to determine morphometric characteristics of composite surface: total surface porosity, area and perimeter of pores.

Surface of TFE/VDF-HA framework was investigated with a Philips SEM 515 scanning electron microscope. Framework surface was coated with thin carbon layer to reflux load and to obtain qualitative images of the framework.

Inner structure of framework micropores was investigated by the method of atomic force microscopy using a «C3M Solver HV» device. Investigations were carried out in the contact mode of microscope work.

Chemical composition of the framework was investigated by means of X-ray fluorescent analysis with the Shimadzu XRF 1800 plant. Specimens (samples) for investigation in form of discs with diameter of 5 mm were fabricated by method of cold pressing of material.

Modification of various substrate surface were performed by the method of RF magnetron sputtering of thin calcium phosphate films with aim to study influence of CaP coatings on properties of these materials. Influence of surface modification by the RFMS was investigated using a «Solver-HV» (NT-MDT) atomic force microscope (AFM), allowing to measure surface relief, its phase contrast, surface potential distribution by Kelvin method (Mironov V.L., 2004; McCaig et al., 2005). Measurements were carried out on air in standard conditions in semicontact work mode with the use of two-pass technique. For working in semicontact mode we used cantilevers of grade NSG11 with needle rounding radius of 10 nm and alloying admixture concentration of $5 \times 10^{20} \text{ cm}^{-3}$. Let's note that this kind of investigations required high cleanness of the surface to be investigated therefore to execute these requirements we prepared some types of reference specimen conditionally divided on groups: I, II, III, IV. Results of influence of RFMS Ca-P coating on reference specimen allow extrapolating obtained data on composite scaffolds.

Specimen of the I group presented plates manufactured from titanium alloy of grade BT - 6 with sizes 20×20×3 mm, which preliminary were polished by mechanical way using GOI paste.

Specimens of the II group were prepared in the same way that specimens of the I group, at this on one side of specimen was applied Ca-P coating formed by method of radio frequency magnetron sputtering (RFMS) of hydroxyapatite target. To apply CaP coating we used the industrial plant «Katod 1M», which vacuum chamber contains standard high frequency magnetron source supplied by HF generator with maximal power 4 kW and work frequency 13.56 MHz. The following technological modes have been selected to apply CaP coating: preliminary pressure in chamber - $5 \cdot 10^{-5}$ Pa, work pressure of Ar - $3 \cdot 10^{-1}$ Pa; specific HF power ~ 20 W/cm²; time of applying - 2 hours. Specimens - witnesses (polished silicon plates with masked part) were used to determine coating thickness, which was determined with a Talysurf 5 (Tyler-Hobson, England) mechanical profilometer by "step" method. With selected sputtering parameters thickness of formed CaP coating was 0.8 ± 0.02 μ m.

Specimens of the III group were prepared in the same way that specimens of the I group, at this on one side of specimen was coated with solution of copolymer of tetrafluorethylene with vinyliden fluoride (TFE/VDF) in acetone by the method of pneumatic spraying. Part of a specimen has been masked to obtain coating - substrate interface. Then, specimens with applied polymer coating were places in the ITM 50.1100 (ITM, Tomsk, Russian Federation) furnace of chamber type, where they were heated up to temperature 200 °C to remove solvent remnants and finally to form coating. Thickness of coatings was determined with a Talysurf 5 mechanical profilometer with the "step" method. Thickness of formed polymer coatings was 5 ± 0.4 μ m.

Specimens of the IV group were prepared in the same way that specimens of the III group, after that surface of formed TFE/VDF polymer was underwent modification by means of forming of calcium phosphate coating by the RFMS method in the same technological modes, as specimens of the II group. At selected sputtering parameters thickness of formed CaP coating was 0.8 ± 0.02 μ m.

AFM images of surface relief, phase contrast and surface potential have been built for all studied types of specimens. Processing of obtained AFM images were performed with help of Gwiddion 2.25 program complex. The following parameters of surface roughness have been determined: mean arithmetic deviation of surface profile Ra , height of profile irregularities by ten points - Rz , maximal height of roughness profile - Pt , mean maximal depth of roughness hollows - Rvm , mean maximal height of roughness peaks - Rpm , mean arithmetic deviation of potential profile φ_a , mean value of surface potential U_m , maximal value of surface potential U_{max} , minimal value of surface potential U_{min} .

To determine limiting water wetting angle of modified surfaces we used the static method of "laying drop". Measurements of limiting wetting angle have been carried out in 60 seconds after placing of a drop on substrate surface. Limiting wetting angle has been accepted as mean value of 5 measurements, which have been carried out with help of an «EasyDrop DSA-15E» (the firm KRUSS) device for drop form analysis.

Toxicological tests, estimations of local irritant action, hemolytic activity of framework from composite materials have been carried out after its sterilization with ethylene oxide in a AN4000 (Andersen Sterilizers Inc.) sterilizator in accordance with GOST R ISO 10993.

Biocompatibility and bone forming ability of framework have been estimated *in vivo* by means of ectopic bone formation test (US Department of Health and Human Services, Web Site) on 40 mice males of BALB/C line with mass of 18–21 g. Titanium discs from alloy BT-6 with diameter $d = 1$ mm with framework applied on their surface were implanted in mice subcutaneously. Before implantation the discs with composite framework had been coated with bone marrow column extracted from animal femur (1.5×10^6 cell/ml), in DI-MEM (ISN) medium with 10 % fetal calf serum («Vector», Novosibirsk, Russian Federation). After 1.5 months the animals were sacrificed with ether anesthesia, the discs were explanted, decalcified, after that paraffin sections (thickness of 10 μ m) of tissue grown on the discs were prepared for further investigation. The sections were painted with hematoxylin-eosin and subjected to histological analysis.

3.2 Results and discussion

XRD spectra of hydroxyapatite powder obtained by means of high temperature burning of biological raw materials at temperature 800 °C are given in Figure 8. Results of investigations of phase composition, crystalline lattice parameters, mean size of hydroxyapatite particles, obtained at various temperatures are given in the Table 1.

Annealing temperature, °C	Revealed phases	Volume phase content, %	Lattice parameters	Average particle size, nm	$\Delta d/d \cdot 10^{-3}$
600	Ca ₅ (PO ₄) ₃ (OH) hydroxyapatite	63.43	A=9.4119 C=6.8756	64	0.9
	Ca ₁₀ (PO ₄) ₆ (OH) ₂ monoclinic hydroxyapatite	36.57	A=9.5189 B=18.7480 C=6.8903	14	2.5
800	Ca ₅ (PO ₄) ₃ (OH) hydroxyapatite	83.0	A=9.4126 C=6.8769	67	0.7
	Ca ₁₀ (PO ₄) ₆ (OH) ₂ monoclinic hydroxyapatite	17.0	A= 9.3190 B=18.4729 C=6.7840	30	2.2
1000	Ca ₅ (PO ₄) ₃ (OH) hydroxyapatite	96.96	A=9.4125 C=6.8763	134	0.4
	Ca ₁₀ (PO ₄) ₆ (OH) ₂ monoclinic hydroxyapatite	3.04	A=9.4623 B=18.7480 C=6.9641	42	1.1

Table 1. Phase composition of hydroxyapatite in dependence of annealing temperature

It has been stated that temperature of raw material treatment influences on phase composition of obtained hydroxyapatite since the increase of treatment temperature decreases output of hydroxyapatite with monoclinic structure. It is shown that the increase of annealing temperature leads to the increase of grain size of obtained calcium phosphates. Grain size of phase Ca₅(PO₄)₃(OH) increases on 200% at the increase of treatment temperature on 400 °C, and grain size of phase Ca₁₀(PO₄)₆(OH)₂ – on 300% (see Table 1).

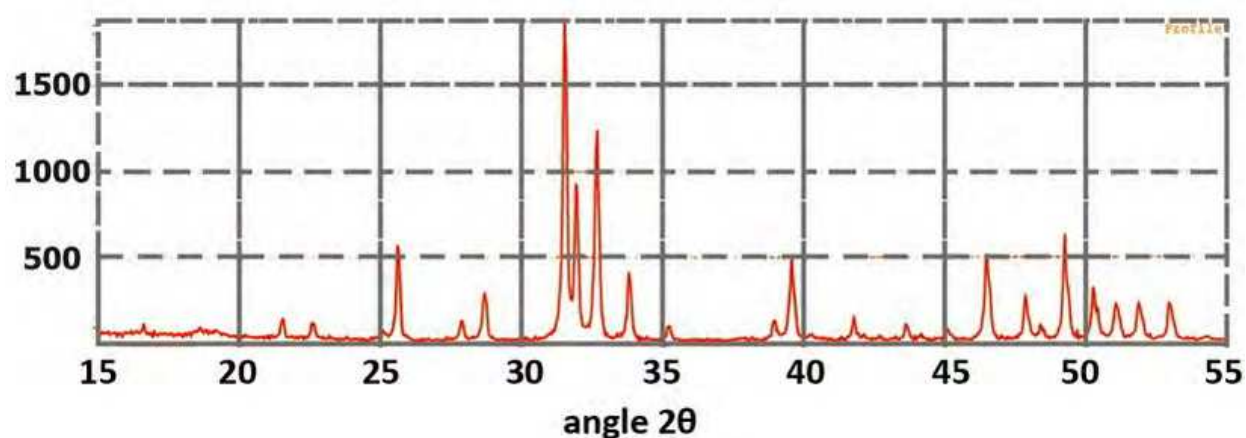


Fig. 8. XRD spectra of hydroxyapatite powder

Images of scaffold obtained in reflected light with help of the “Motic” optical microscope at various amplifications are given in Figure 9.

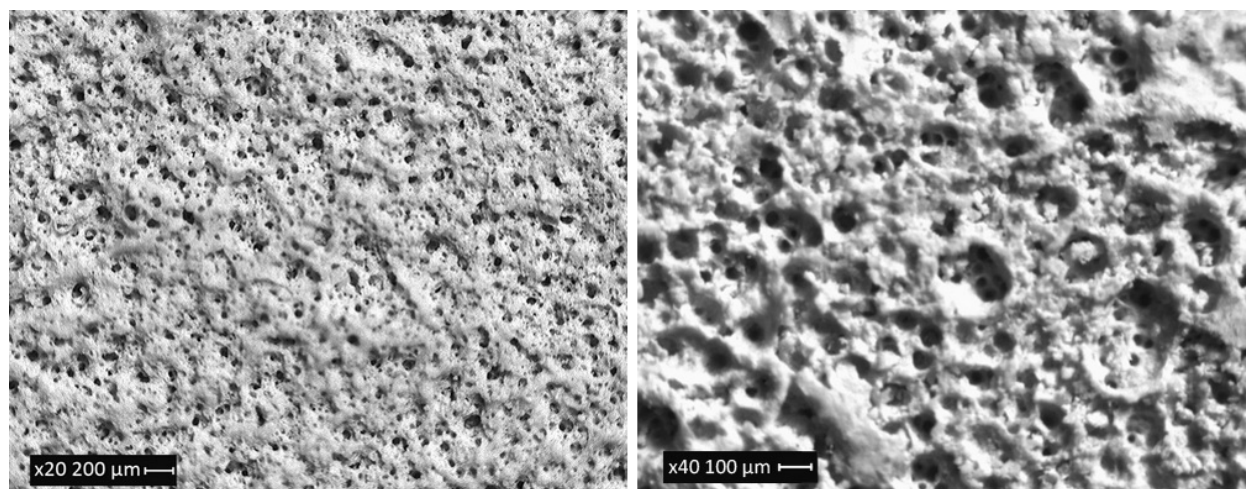


Fig. 9. Image of scaffold surface at various amplifications

The main morphometric characteristics of framework surface obtained by means of computer processing of optical images with help of the Motic Image Life plus program package, are following: total pore area 441280 μm^2 , total porosity of the framework 47.79 %, min pore area 0.8 μm^2 , max pore area 25379.6 μm^2 , mean pore area 190.0 μm^2 , min pore perimeter 4.7 μm , max pore perimeter 3393.1 μm , mean pore perimeter 22.7 μm . Histogram of pore distribution by sizes is given in Figure 10.

Analysis of obtained data allows distinguishing at least three conditional levels of TFE/VDF-HA framework: porosity with pore sizes about 10 μm^2 , microporosity with pore sizes under 5000 μm^2 and macroporosity with pore sizes more than 8000 μm^2 . To our opinion presence of some porosity levels says about presence of some mechanisms of pore formation that will be investigated later. The most contribution on total surface porosity (TSP) is put by micropores with sizes more than 5000 μm^2 .

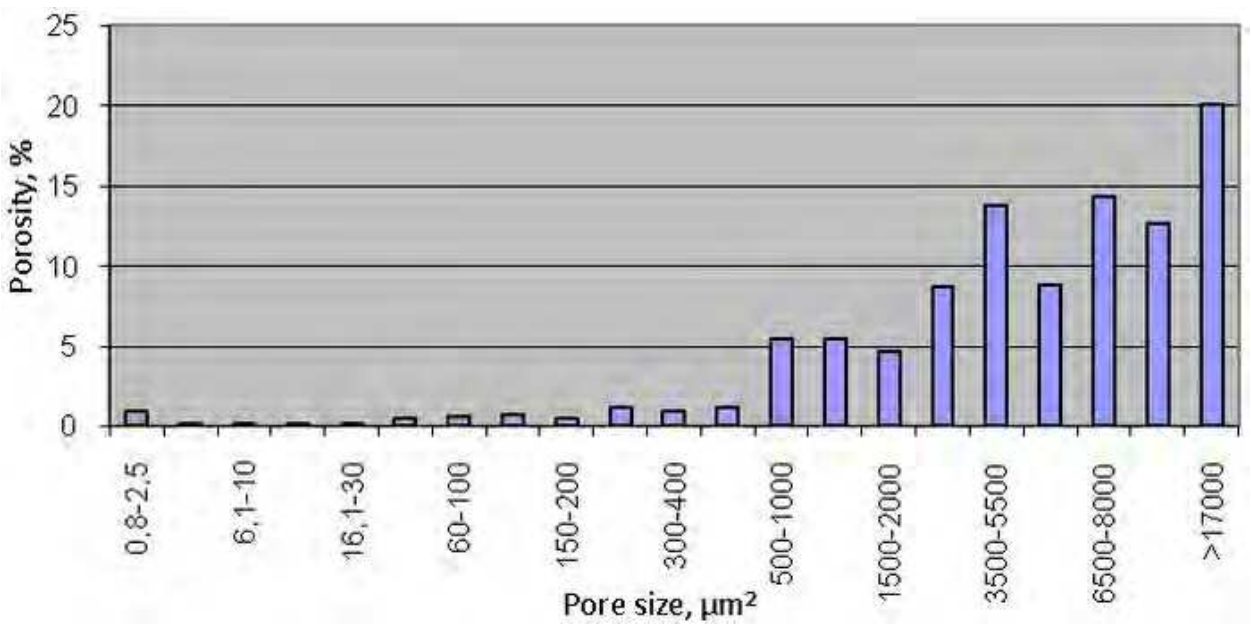


Fig. 10. Histogram of pore distribution

Images of scaffold obtained with the help of scanning electron microscope at various amplifications are presented in Figure 11.

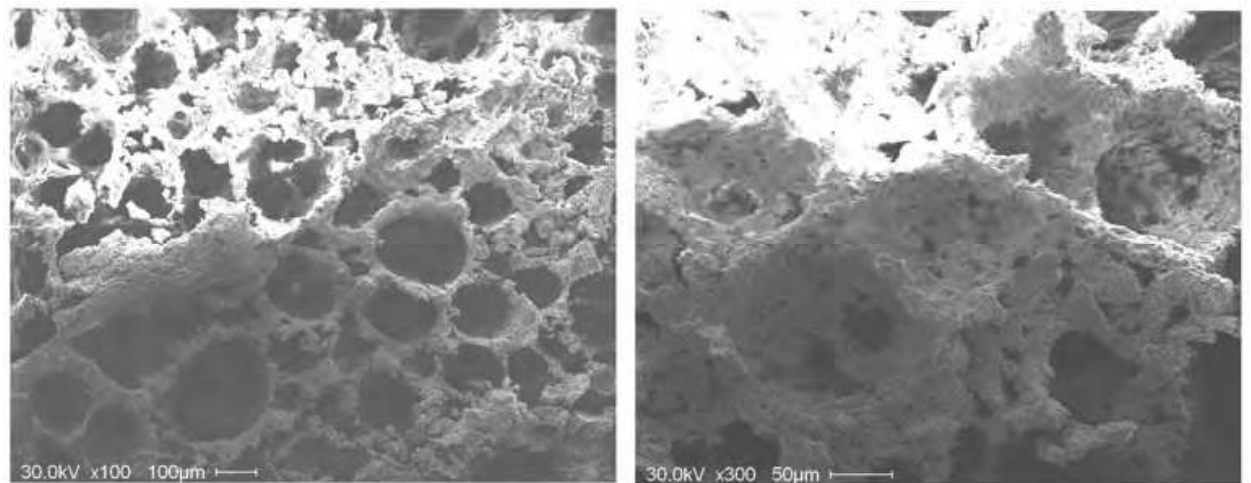


Fig. 11. Scanning electron microscopic (SEM) image of scaffold surface at various amplifications

The analysis of framework surface carried out by scanning electron microscopy had allowed to verify that used obtaining modes made it possible to steadily create porous surface with pore diameter more than 100 μm forming multilevel interpenetrate structures. Sufficient magnification clearly shows that the framework microstructure is a porous system in which particles of HA are interconnected with polymer binding agent. Images of inner structure of scaffold macropores obtained with the help of a «C3M Solver HV» atomic force microscope are presented in Figure 12. This method allowed determining that macropore walls are penetrated with the system of smaller pores with size approximately 0.5 μm . It can be assumed that the walls and the micropores have a similar structure.

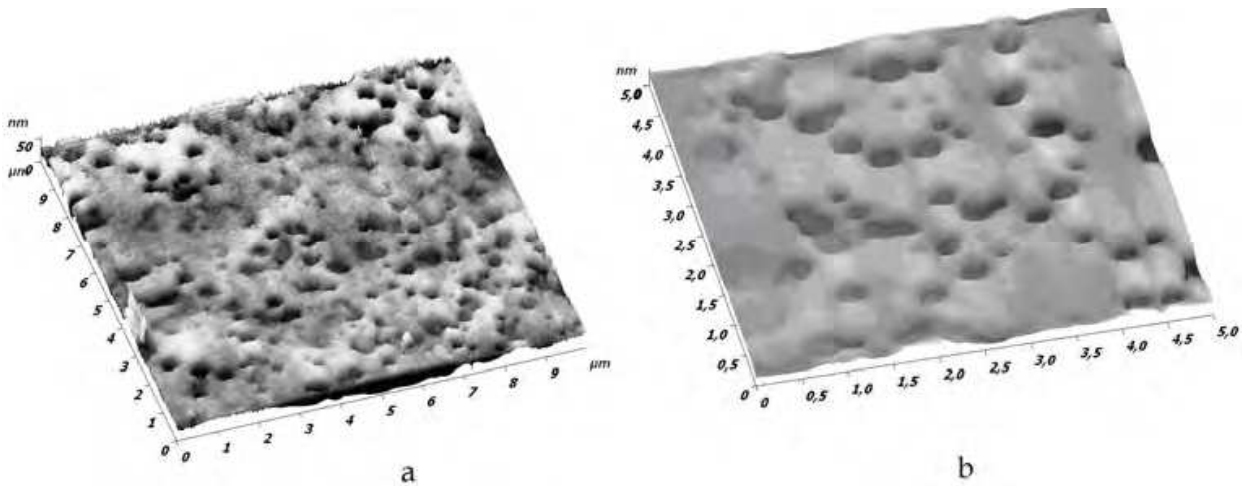


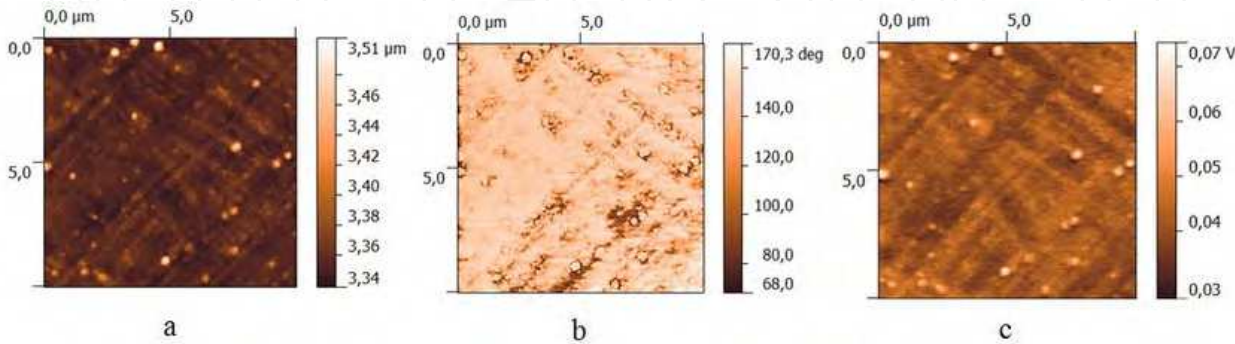
Fig. 12. AFM images of inner structure of scaffold macropores, a – 10×10 μm, b – 5×5 μm

Thus microscopic investigations of scaffold structure show that it is similar to cancellous bone structure. It is the main factor promoting colonization of frameworks with osteoblasts and pullulation of bone tissue in its pores with formation of bone blocks of the “biocomposite – bone tissue” type that leads to increasing of fixation rigidity, for example, of intramedullar implant in medullary channel (Karlov & Shachov, 2001). Data of scaffold chemical composition obtained by means of X-ray fluorescent analysis are given in the Table 2.

Ca	P	O	F	Mg	K	Na	Ni	others
37.1635	16.3777	32.6303	11.7968	0.6010	0.0444	0.5422	1.9413	0.8441

Table 2. Chemical composition of composite scaffold, mass %

Analysis of element composition shows, that the main composition of composite scaffold is calcium, phosphorous, fluorine and oxygen. Material contains admixtures of magnesium, nickel, potassium, carbon as well as trace quantities of copper, iron and sodium. Presence of admixtures can be explained by the fact that hydroxyapatite obtained by means of burning of biological raw materials has not ideal crystalline lattice with admixtures of various elements (Barinov & Komlev, 2005).



a – surface relief image, b – phase contrast image, c – surface potential images

Fig. 13. AFM surface images of the I group specimens, scanning field is 10×10 μm,

AFM images of surface of I group specimens – titanium are presented in Figure 13. On the presented images of surface relief (see Fig. 13, a), phase contrast (see Fig. 13, b) and surface potential (see Fig. 3, c) it is possible to separate globular defects being, by all appearance, particles of abrasive, “inserted” in titanium surface in process of its polishing, however detailed investigation of this question is matter of additional investigations.

Results of measurement of polished titanium substrate obtained using a Gwiddion 2.25 program complex of AFM images are presented in the Table 4. Measured parameters of surface roughness of the I group specimens allow to attribute obtained surfaces to the 13th class of roughness (See Table 3). Results of measuring of surface potential parameters of the I group specimens are presented in the Table 4.

Analysis of obtained data allows say about practically zero potential of the I group specimens, unessential changes of potential in our opinion are connected with adsorption of water vapors by a substrate and unessential oxidation of a substrate.

Images of surface relief (a), phase contrast (b) and surface potential relief (c) specimens of polished titanium with CaP coating – the II group are presented in Figure 14. Analysis of obtained images allows making a conclusion that CaP coating applied on a polished titanium substrate by the RFMS method essentially changes surface microrelief, on images of relief and phase contrast it is easily to trace grain boundaries with sized about 0.3 μm and height 0.19 μm that is connected with mechanisms of atomic growth of CaP coatings. Analysis of phase contrast allows to make conclusion that coating covers a substrate totally, isolating it from external action. Investigation of surface potential of CaP coating, gives evidence about its increasing toward positive values that is displayed especially brightly on coating projections presenting centers of crystallization and growth of thin CaP film.

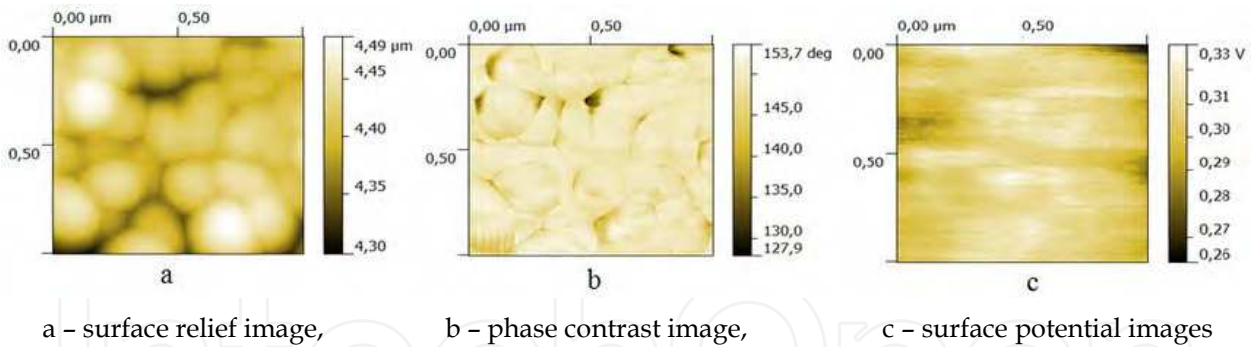


Fig. 14. AFM surface images of the II group specimens, scanning field is 1×1 μm,

Results of measurements of roughness of the polished titanium substrate with CaP coating are presented in the Table 3. On the basis of this data it is possible to make conclusion that CaP coating changes essentially microrelief of the polished titanium substrate, parameters of its roughness increase; obtained coatings are attributed to the 10th class. Increasing of coating roughness in micro scale can be additional stimulating factor of tissue growth, for example, by means of attachment and proliferation of osteogenic cells on CaP coating surface (Karlov & Khlusov, 2003).

Let’s note that CaP coatings formed on a titanium substrate by the RFMS method increase mean values of surface potential on value up to 0.052 V. At this in addition to fields with positive surface load, one found microfields with negative surface potential with value up to -0.395 V in relation to ground.

Specimen group	Mean arithmetic deviation of surface profile <i>Ra</i> , nm	Height of profile inequalities by ten points <i>Rz</i> , nm	Maximal height of roughness profile <i>Pt</i> , nm	Mean maximal depth of roughness holes <i>Rvm</i> , nm	Mean maximal height of roughness peaks <i>Rpm</i> , nm
I	6.32	96.1	95.97	12.47	38.82
II	152	801	1622	440	429
III	16.2	92.2	231.3	52.5	49.9
IV	47.2	376	918.2	141.2	152.2

Table 3. Roughness parameters of specimens by AFM data

Image of surface relief, phase contrast and surface potential of the III group specimens – titanium with polymeric TFE/VDF coating is given in Figure 15. Analysis of AFM images allows saying that formed surface of polymer TFE/VDF coating is sufficiently uniform film with negative surface potential. TFE/VDF coating film is formed as ordered structure of polymer spherulites presenting polymer parts with various degree of crystallinity that corresponds to modern ideas about structure of TFE/VDF copolymers (Panshin et al., 1978).

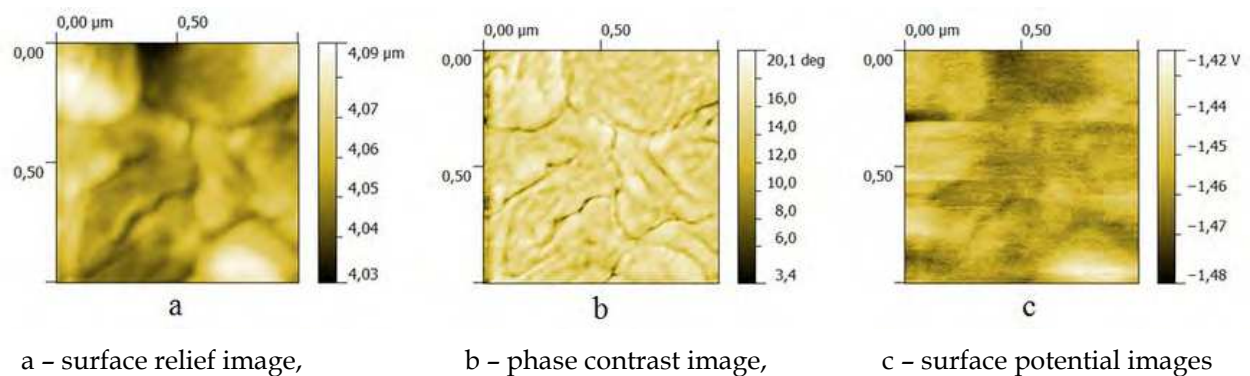


Fig. 15. AFM surface images of the III group specimens, scanning field is 1×1 μm,

Results of measurements of surface roughness of TFE/VDF polymer coating are presented in the Table 3. Analysis of obtained data testifies that polymer coating allows to level defects appearing in process of mechanical treatment of a titanium substrate, obtained coatings are attributed to the 13th class. Such coatings can be used as biomaterials with low ability to cell adhesion, for example, thromb resistive coatings of vascular stents for which is necessary together with high class of surface cleanness and negative surface potential. It is known that complex of these requirements provides reliable functioning of intravascular stents with minimal percentage of secondary operative interventions (Guidance, 2003).

Results of measurement of surface potential parameters of the III group of specimens are presented in the Table 4.

AFM images of surface relief (a), phase contrast (b) and surface potential relief (c) of the IV group specimens – titanium with TFE/VDF polymer coating modified with RF sputtering of CaP coating are presented in Figure 16. Coating covers totally polymer layer that is proved by distribution of phase contrast. It has been stated that CaP coatings on a polymer substrate formed by RFMS of hydroxyapatite target resemble, in many way, coatings formed on a polished titanium substrate and present uniform conglomerate consisting from separate

grains of calcium phosphates with size about 0.3 μm. It is displayed especially well on image of phase contrast of CaP coating. On the basis of above mentioned, it is possible to suppose that substrate material where RFMS coating is formed, does not act essential influence on CaP coating parameters.

Specimen group	Mean arithmetic deviation of potential profile φ_{ar} , V	Mean value of surface potential U_m , V	Minimal value of surface potential U_{min} , V	Maximal value of surface potential U_{max} , V
I	0.00392	0.03311	-0.0092	0.0655
II	0.133	0.052	-0.395	0.642
III	-0.082	-1.82	-2.178	-1.272
IV	-0.042	-0.526	-1.014	-0.054

Table 4. Surface potential parameters of specimens by AFM data

The analysis of images of surface potential of CaP coating and data of Table 4 demonstrates essential decrease of negative potential of initial polymer substrate after applying of CaP coating. Possibly it is connected with the fact that ceramic-like CaP coatings is semiconductor of p-type with large width of energy band gap equal about 4 eV (Rosenman & Aronov, 2006). This circumstance is stated by the method of exoelectron emission being sensitive to presence of defects in thin surface layer. Using of this method allowed to state that nanostructured calcium-phosphate surface has many defects. Moreover, part of defects are the centers of electron – hole capture bearing electrical charge. Owing to various mobility of electrons and holes these localized charges take part in formation of double electric layer and can change surface potential (Aronov & Rosenman, 2007). In general, we can conclude that CaP coatings formed by the RFMS have positive surface potential that is conformed to article (Khlusov et al., 2011).

Results of measurement of roughness of polymer TFE/VDF coating with CaP coating formed by the RFMS method – the IV group of specimens - are presented in the Table 3.

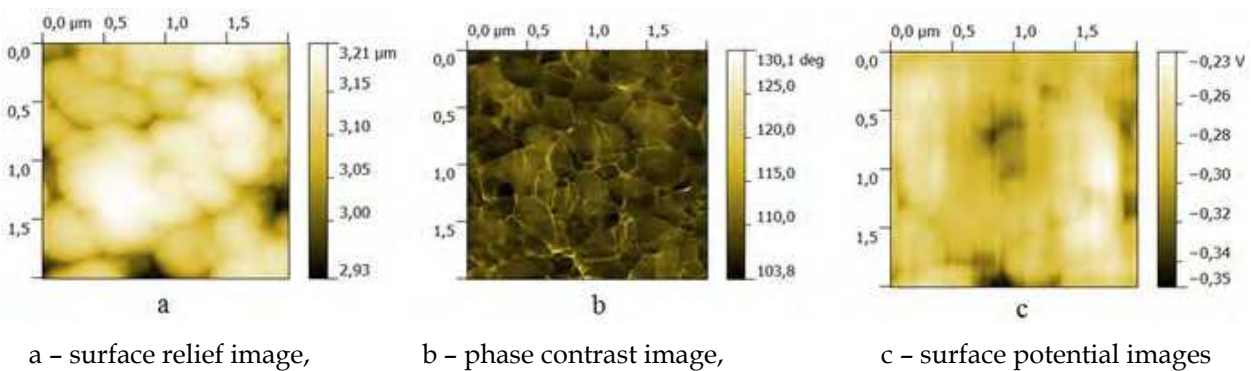


Fig. 16. AFM surface images of the IV group specimens, scanning field is 2×2 μm,

On the basis of coating roughness data given in the Table3, it is possible to make conclusion that CaP coating changes essentially microrelief of polymer coating as in the case of a polished titanium substrate. Value of mean arithmetic deviation of surface profile *Ra* increases in 2.9 times, at maximal height of roughness surface profile *Pt* increases in 3.9 times. Formed coatings are attributed to the 11th roughness class.

Photo of water drop placed on surface of TEF/VDF coating applied on polished titanium surface is presented in Figure 17. Mean value of limiting wetting angle of TEF/VDF copolymer surface θ measured with the “EasyDrop” device was 95.5° that gives evidence of high hydrophobicity and low surface energy of TEF/VDF copolymer.

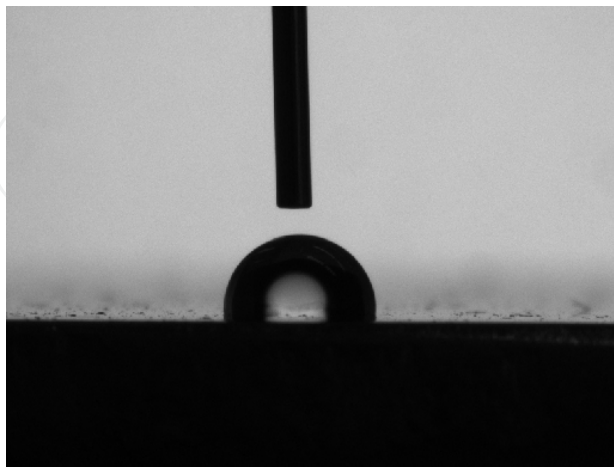


Fig. 17. Water drop form on surface of TFE/VDF copolymer

Photo of water drop placed on surface of TEF/VDF coating modified by means of RFMS of hydroxyapatite target in 2 hours is presented in Figure 18. Mean value of limiting wetting angle of TEF/VDF copolymer with calcium phosphate coating was 55.1° that gives evidence of the increase of the free energy of modified TEF/VDF copolymer surface and the increase of its hydrophilicity.

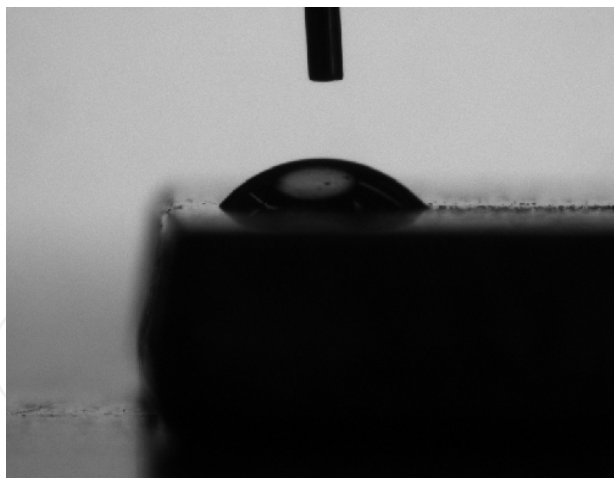


Fig. 18. Water drop form on surface of TFE/VDF copolymer, modified with the RFMS method

Thus modification of polymer TFE/VDF material surface realized by the method of RFMS hydroxyapatite target allows to increase surface energy of TFE/VDF polymer and to put it hydrophilic properties. Extrapolating this conclusion on the scaffold manufactured on the basis of TFE/VDF copolymer and hydroxyapatite we can suppose that modification of scaffold surface by the RFMS method should allow to range limiting wetting angle that must promote its impregnation of various drugs.

Data of toxicity, local irritant action, apirogenity and sterility of composite scaffold obtained in accordance with ISO 10993 are given in the Table 5. In the course of investigation death of laboratory animals did not registered, macroscopic changes of organs and tissues, and changes of weight coefficients of inner organs have not been revealed. Drawings from composite framework did not render local and general irritant action on skin and mucosa membrane of laboratory animals.

№	Index name	Admissible values	Test results	Conclusion of conformity
1.1	Toxicological tests Irritant action on skin and mucosa membranes of animals in balls : Skin Mucosa of rabbit eye	0 0	0 0	Conforms Conforms
1.2	Acute toxicity at abdominal injection: - Mortality rate Clinical symptoms : -Macroscopic changes of organs and tissues; -Weight coefficients of inner organs (presence of trusted changes)	No No No	No No No	Conforms Conforms Conforms
3	Determination of hemolytic activity	Not more than 2%	0.7	Conforms
4	Determination of toxicity index	70-120%	88.4%	Conforms
5	Determination of pyrogenity	Raise of temperature not more than 3°C	0.4 °C	Conforms
6	Microbiological index	Sterile	Sterile	Conforms

Table 5. Results of investigations of composite scaffold cytotoxicity

Results of investigation of composite scaffold *in vivo* after subcutaneous implantation in mice of BALB/C line is presented in the Table 6.

Inflammation in implantation site	Encapsulation of implant	Tissue plate, %	Histological estimation	Efficiency of bone tissue growth, %
No reaction	Low reaction	100	Bone with bone marrow	93

Table 6. Quantitative estimation of biological activity of composite scaffold

In the course of investigation it has been noted that animals endured easily surgery intervention. There were not pointed out natural death of animals, local or general inflammation and toxic reaction on implant, and scaffold biocompatibility was good. Framework surface has thin stromal capsule. Histological section of obtained preparation is presented in Figure 19.

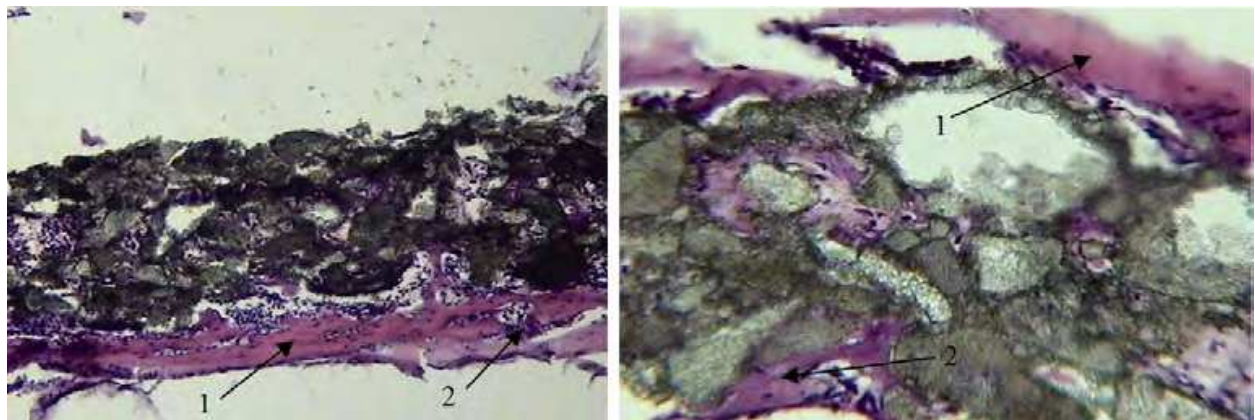


Fig. 19. Histological sections of preparation grown on composite scaffold, painting – hematoxylen – eosin, 1 – bone tissue, 2 – medullary cavity, filled with bone marrow

On histological section (Fig. 19) one can see bone tissue (1) and medullary cavities filled with bone marrow (2). Ingrowth of bone in composite scaffold pores is observed that testifies possibility its application for osteogenesis.

4. Conclusion

Method of thermal induced phase separation allows to obtain high porous scaffolds with interconnected porosity necessary to provide processes of osteoinduction and osteoconduction on the basis of tetrafluorethylene with vinyliden fluoride copolymer and hydroxyapatite (TFE/VDF - HA).

The method of high temperature burning of biological raw materials with following multiply washing and drying allows obtaining hydroxyapatite used as biologically active filler for composite scaffolds.

Chemical composition of composite scaffold on the basis of tetrafluorethylene and vinyliden fluoride copolymer and hydroxyapatite (TFE/VDF - HA) is presented mainly by calcium, phosphorous, oxygen and fluorine. Qualitative ratios of elements in composites depend on share of hydroxyapatite added to polymer. Mass ratio $\text{Ca/P} = 2.27$ does not depend on quality of hydroxyapatite in composite but is determined by chemical composition of initial HA.

Method of radio frequency magnetron sputtering of hydroxyapatite target allows modifying surface of composite scaffold by effective way. It is shown that modification of composite scaffold surface by the RFMS method increases surface roughness that is stimulating factor for attachment and proliferation of osteogenous cells.

It was shown by the Kelvin method that CaP coating formed by the RFMS of hydroxyapatite target changes surface potential of a scaffold moving it in the field of positive values in relation to ground.

Modification of polymer scaffold surface by RFMS would allow ranging its limiting wetting angle that must provide its ability to be impregnated with various drugs.

Proposed scaffolds after sterilization with ethylene oxide are nontoxic, apirogenous and sterile.

Tests *in vivo* have not revealed negative tissue reaction on implanted scaffold. Test of ectopic bone formation demonstrates positive result of implantation.

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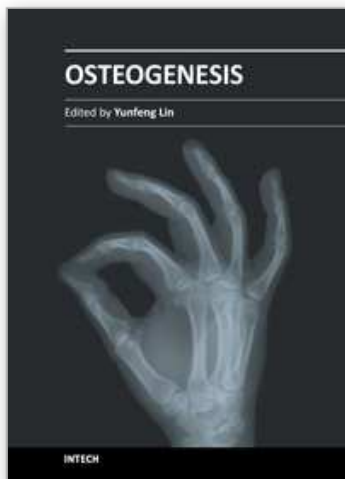
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